SCREENING FOR INHIBITOR OF PROTEIN KINASE FROM MICROBIAL AND PLANT EXTRACTS

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DECLARATION

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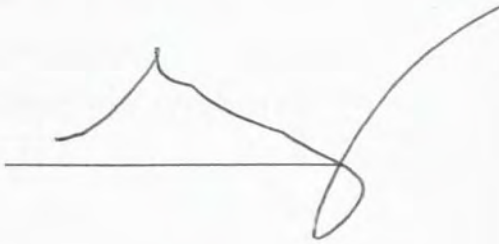

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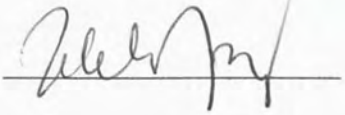

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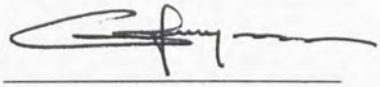

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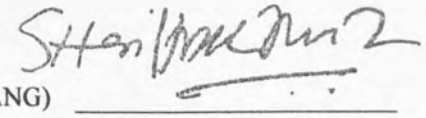

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ABSTRACT

Microbial and plant extracts are productive sources of new bioactive metabolites for drug discovery. Screening for secondary metabolites is an established method to identify novel biologically active molecules. The screening system used in this study was yeast-based screening system. The glycogen synthase kinase-3 beta (GSK-3β) and mitogen-activated protein kinase kinase (MAPKK) were the molecular targets of this study since these kinases play a vital role in tumorigenesis, apoptosis, and cell proliferation. Mutant yeasts strain $MKK1^{P386}$ and H10075 were used as models for searching MAPKK and GSK-3β inhibitors. The acetone extract of actinomycete strain H7667 showed GSK-3β inhibitory activity but not against MAPKK inhibitory activity. The active acetone extract of H7667 was dried and subjected to liquid-liquid extraction with butanol and aqueous at ratio of 1:1. The obtained butanol extract of H7667 gave five significant peaks with retention time at 4.5 min, 18.4 min, 19.0 min, 21.4 min and 23.5 min respectively as analysed using RP-HPLC. The fractions of this extract showed negative results against the GSK-3β possibly due to small amount of bioactive compounds after fractionation. Five of the 27 plant extracts (crude, chloroform, methanol: aqueous and *n*-butanol extracts of *Allium cepa*, and aqueous extract of *Tinospora crispa*) had shown potent inhibitory activity against the MAPKK, whereas the *Piper nigrum* crude and chloroform extracts had shown to be potential inhibitors against GSK-3β. The *n*-butanol extract of *Allium sativum* showed toxicity against both MAPKK and GSK-3β. The *Allium cepa*, *Tinospora crispa* and *Piper nigrum* are potent anti-MAPKK and anti-GSK-3β agents which can be further developed as drugs for cancer and Alzheimer's diseases treatments.

PENYARINGAN PERENCAT KINASE PROTEIN DARIPADA EKSTRAK MICROBIAL DAN TUMBUHAN

ABSTRAK

Ekstrak mikrobial and tumbuhan merupakan sumber bagi metabolit bioaktif untuk penemuan ubat-ubatan baru. Penyaringan metabolit sekunder merupakan satu kaedah yang telah digunakan untuk mengenalpasti molekul yang bioaktif dan baru. Sistem penyaringan yang digunakan dalam kajian ini adalah berdasarkan sistem yis. GSK-3β dan MAPKK dipilih sebagai sasaran pada peringkat molekular kerana mereka memainkan peranan yang penting dalam tumorigenesis (pertumbuhan tumor), apoptosis (pemicu kematian sel) dan proliferasi sel. Strain yis mutan MKK1^{P386} dan H10075 digunakan dalam sistem penyaringan perencat-perencat MAPKK and GSK-3β masing-masing. Ekstrak aseton bagi strain actinomycete H7667 menunjukkan aktiviti perencatan dalam sistem penyaringan perencat GSK-3β tetapi ia tidak menunjukkan aktiviti perencatan dalam sistem penyaringan perencat MAPKK. Ekstrak aseton H7667 dikeringkan dan diekstrak dengan pengekstrakan cecair-cecair pada nisbah butanol:akueus (1:1). Ekstrak butanol ini dianalisis dengan RP-HPLC di mana lima puncak yang signifikan didapati pada masa retensi 4.5 min, 18.4 min, 19.0 min, 21.4 min and 23.5 min masing-masing. Fraksi-fraksi ekstrak ini tidak merencat GSK-3β disebabkan oleh amaun kompoun bioaktif yang sedikit selepas fraksinasi. Lima daripada 27 ekstrak-ekstrak tumbuhan (ekstrak mentah, ekstrak-ekstrak kloroform, methanol: akueus dan *n*-butanol *Allium cepa*, dan ekstrak akueus *Tinospora crispa*) merupakan perencat MAPKK yang berpotensi. Ekstrak mentah dan ekstrak kloroform *Piper nigrum* berpotensi menjadi perencat GSK-3β. Ekstrak *n*-butanol *Allium sativum* adalah tosik terhadap MAPKK dan GSK-3β. *Allium cepa*, *Tinospora crispa* dan *Piper nigrum* berpotensi menjadi agen-agen anti-MAPKK dan anti-GSK-3β yang sesuai diperkembangkan sebagai ubat-ubatan untuk rawatan cancer dan penyakit Alzheimer.

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LIST OF SYMBOLS, UNITS AND ABBREVIATION

DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
GSK-3β	Glycogen Synthase Kinase-3 beta
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEK	MAP kinase/ERK kinase
SC-Ura	Synthetic complete minus uracil
g	Gram
mg	Milligram
$mgml^{-1}$	Milligram per millilitre
mL	Millilitre
M	Molar
mM	Millimolar
µm	Micrometer
µl	Microlitre
nm	Nanometre
gml^{-1}	Gram per millilitre
°C	Degree Celsius
w/v	Weight over volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

All known cultures from ancient times to the present day have used plants as a source of medicines. According to the world Health Organization (WHO), nearly 70% of the world's population use medicinal plant remedies, especially those in the developing and under-developed countries (Shamsul Khamis *et al.*, 2003). In those countries plants are sometimes used as sources of direct-therapeutic agents because they are easily and cheaply available and modern medicine could be expensive.

However, in ancient times, the traditional medicine practitioners were using plants to cure illness and diseases, such as asthma, fever, eye sore, cancer, kidney problem and so on, without knowing the chemical ingredients in plants. Nowadays, with the development of science and technology, scientists are working on isolation and characterisation of new compounds from plants. They also study on bioactivities of crude extracts and purified chemical compounds. Thus, the herbal medicine has regained its popularity as a complementary or alternative medicine and is gaining world acceptance. With the increase in the popularity of the plants as an alternative medicine, more herbal drugs are now available in various forms in the market. They

include tablets, capsules and tea- bags as well as in the form of chips and fragments for boiling. For example, Diosgenin, a compound found in wild yam, has been used as the primary chemical base for the synthesis of many pharmaceutical steroidal drugs. According to the latest reports, there are about 1200 species of plants in Malaysia that have potential pharmaceutical value. Plans have also been drafted to develop Malaysia into one of the largest global producer of herbal raw materials and products by 2010 (Khatijah Hussin, 2006).

Even though much of the emphasis has been based on traditional medicinal plants, the microorganisms do not loose their important role as a source of medicine. Microorganisms have produced secondary metabolites for microbial interactions or defence substances to fight other organisms. Some of these secondary metabolites are containing biologically active compounds, which can act as antibiotics. For instance, the invisible microbes like filamentous bacteria, actinomycetes, mycobacterium and fungi, have produced most of the important antibiotics and other pharmaceutical compounds. Thus, a biological approach has been developed to capture the biodiversity of the actinomycetes and microfungi from the forests of Malaysia, particularly Sabah. This approach is developed to isolate as many different (10 000) strains of actinomycetes and fungi, so that a few of them (less than 10) would produce the desired secondary metabolites against the selected molecular targets in the screening programme for compounds against tuberculosis, cancer and memory loss (Ho, 2003).

Recently, signal transduction, the transmission of signals within the cell, becomes one of the most important aspects of research on diseases. All cells response

to environmental signal. The binding of extracellular stimulus such as growth factors and hormones to the receptor can stimulate intracellular responses that including cell proliferation, differentiation, gene transcription, metabolism, motility and apoptosis. Most of diseases such as cancer and diabetes are caused by the failure in function of signal transduction pathway. Commonly, signal transduction pathways in eukaryotes regulate protein kinases that phosphorylate and control the activity of proteins involved in metabolic and transcription events (Sitatamayya, 1999). Deregulated of those protein kinases activities is a cause of disease. Due to protein kinases play an essential role in virtually all cellular processes and in most diseases, extensive searches for selective inhibitors of these enzymes have been carried out.

1.2 Objectives of Study

The following are the objectives of this study:

a. To screen for inhibitor against mitogen-activated protein kinase kinase (MAPKK) from microbial and plant extracts.
b. To screen for inhibitor against glycogen synthase 3-beta (GSK-3β) from microbial and plant extracts.
c. To fractionate the active microbial extract

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This literature review encompassed the actinomycetes and plant samples used in this research. This chapter would latter delve into signal transduction, protein kinase, MAPK cascade, GSK-3β and inhibitors of MAPK and GSK-3β.

2.2 Actinomycetes

Actinomycetes are bacteria that belonging to order *Actinomycetales*. They are filamentous, gram-positive bacteria that form branching filaments. They contain high composition of guanine and cytosine (GC) of about 63-78% of DNA base composition (Madigan *et al.*, 2003). Their morphology resembles that of filamentous fungi. However, their filaments consist of the prokaryotic cells with the diameter of the filaments are smaller than the fungi (Tortora *et al.*, 2002). They form a ramifying network of filamentous known as mycelium. They will form spore when mature. The manner of the formation is usually used to distinguish between actinomycetes. Actinomycetes are primarily soil organisms

(Madigan *et al.*, 2003). A few of them can be found in aquatic habitats (Lam, 2006). They are also found in mud at the bottom of ponds, lakes and rivers.

Some of the genera of actinomycetes are *Streptomyces*, *Nocardia*, *Gordonia*, *Streptosporangium* and *Rhodococcus* (Tortora *et al.*, 2002). Genus Streptomyces is the most abundant and the best studied actinomycetes. This bacterium is commonly isolated from the soil and produces a gaseous compound called geosimin that gives the fresh soil its earthy odour (Madigan *et al.*, 2003). The aerial filaments of streptomyces are called sporophores. *Streptomyces* form spores (called conidia) at the end of the aerial filaments (Tortora *et al.*, 2002). Mature *Streptomyces* often has pigmented conidia and sporophores. Different types of *Streptomyces* could have different colour of conidia and sporophores. This is the unique characteristic of the *Streptomyces* (Madigan *et al.*, 2003).

2.2.1 Actinomycetes Secondary Metabolites

Actinomycetes produce secondary metabolite to inhibit the growth of other bacteria. They do so for protection of themselves. Actinomycetes are most economically and biotechnologically valuable microorganisms. This is due to the fact that they are responsible for the production of about half of the discovered bioactive secondary metabolites, antibiotics, antitumour agent, and immunosuppressive agents such as bonactin (antibacterial and antifungal), chinikomycins (anticancer) and diazepinomicin (antibacterial, anticancer and anti-inflammatory) (Lam, 2006). About 70% of known drugs have been isolated from actinomycetes bacteria (Medhi *et al.*, 2006). Streptomyces

are the largest producer of secondary metabolites that are valuable in pharmaceutics (Madigan *et al*., 2003).

An important group of secondary metabolites such as immunosupressants FK506 (tacrolimus) (Kino *et al*., 1987), FK 520 (ascomycin) (Hatanaka *et al*., 1988 a) and rapamycin (sirolimus) (Sehgal *et al*., 1975) is produced by *Streptomyces* sp. and *Streptomyces hygroscopicus*, respectively. Actinomycetes may have originally evolved the ability to synthesis and secret FK506 and rapamycin as antimicrobial agents to inhibit the growth of competing microorganisms in the soil (McCabe *et al*., 1986).

2.3 Plants used in the Study

There are 11 plant species studied: *Allium cepa, Allium sativum, Alpinia galangal, Anacardium Occidentale, Calophyllum inophyllum, Centella asiatica, Chromolaena odorata, Piper nigrum, Polyalthia bullata King, Tinospora crispa* and *Zingiber officinale*.

Garlic (*Allium Sativum*) and Onion (*Allium cepa*) are among the oldest of all cultivated plants with their origin in centre Asia. Garlic has been used as spice, food and folklore medicine for over 4000 years, and is the most widely used research medicinal plant (Ali *et al*., 2000). They are widely known for their biological properties. The compounds involved in the biological mechanisms have been identified from them, such as sulphur and seleno compounds. The beneficial effect of garlic on health including

protection against cardiovascular diseases and cancers results from all of these compounds (Arnault and Auger, 2006).

Alpinia galangal is a kind of plant in Zingiberacea family. This plant contains essential oil, flavonoids (e.g. galangin, galangin monomethyl ether, kaempferol and quercetin). In traditional medicine in Malaysia, an infusion of the rhizome is used for treating fever, bronchitis, rheumatism and diabetes (Samy *et al.*, 2005).

Anacardium Occidentale is a plant belonging to Anarcardiaceae family. It contains oleic glyceride, stearic glyceride, anacardic acid, gum-arabic and bassorin. In traditional medicine, the bark is boiled in water and drunk for treatment of diarrhoea, mouth ulcers and diabetes. The oil from the pericarp is applied on warts, cracks on soles of feet, corn and leprous sores (Samy *et al.*, 2005).

Calophyllum inophyllum is a plant belonging to Clusiaceae family. Its leaves and seed contain inophyllum B and P, which are potent non-nucleoside inhibitors of human immunodeficiency virus (HIV) type 1. It also contains 4-phenylcoumarins that may be potential cancer chemopreventive agents. Besides, its flowers contain essential oil which composed of 25 compounds that have antibacterial, anti-inflammatory and phagocytosis stimulant activities. Other chemical constituents include hydroxyl acid, calophyllic acid, hydrocyanic acid and saponin. It has been used to treat rheumatism, burns and inflamed eyes (Samy *et al.*, 2005).

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